mutants, sites previously shown to be involved in BK Ca^{2+}-sensitivity and located closely to D369, also affected channel-PIP_2 affinity. Our results show that the IC_{50} values for neomycin effects on both D362G and D367G were largely decreased (~100-fold), indicating that the mutations weakened channel-PIP_2 interaction. In contrast, the D369G mutation increased the IC_{50} for neomycin in a voltage-dependent manner, suggesting an enhanced channel-PIP_2 interaction. Taken together, these results suggested that mutation of the negatively charged residues D362 and D367, which lowers Ca^{2+} affinity, also decreases channel-PIP_2 affinity, while the mutant D369G, which increases Ca^{2+} affinity also enhanced channel-PIP_2 interaction. Thus Ca^{2+} and PIP_2 affinities are interrelated down to the single site interaction level.

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Structure-Function Studies of the Large Conductance Voltage-and Calcium-Activated Potassium Channel BETA1 Auxiliary Subunit

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BK channels are large conductance, voltage- and calcium-activated potassium channels. In smooth muscle, these channels maintain a polarized negative membrane potential and therefore deactivate calcium influx through voltage-gated calcium channels and reduce contraction. The smooth muscle-specific auxiliary β1 subunit confers increased BK opening and therefore plays an essential role in limiting smooth muscle contraction. β1 has at least two opposing gating effects: reducing intrinsic opening and stabilizing voltage-sensor activation. The goal of this research was to identify residues and structural domains that mediate these gating effects. Here, we performed an Alanine-scanning mutagenesis of β1 residues identical between β1, β2 and β4 based on the assumption that functionally important residues are likely to be conserved among family members. Effects of the Alanine mutants were categorized based on steadystate and kinetic effects on gating at 60, 4 micromolar and nominal 0 calcium. We found that 17 mutants display moderate or no effects at all calcium. 10 Class I mutants reduce conductance-voltage shifts at both high and nominal 0 calcium. Interestingly, all Class I residues are non-polar, and reside in the extracellular loop. This suggests that extracellular key residues may promote opening by providing a non-polar environment to the activated voltage senor. The only Class II mutant, G171A, abolished β1's ability in reducing opening in the absence of calcium. This suggests that G171 plays a critical role in reducing intrinsic opening. Finally, the mutagenesis data suggests that 2 evolutionarily conserved Cysteine pairs and their disulfides may play an important role in the extracellular β subunit structure. Combining single and double mutations, we found that the two amino acid pairs C53-C135 and C76-C103, may serve to form disulfide bonds that bring key extracellular Class I residues in close approximation.

3156-Pos Board B261

Investigation of BK Channel Gating using Mallotoxin

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While large-conductance, BK K^+ channels are activated by both voltage and by Ca^{2+} in the microM range, the channels can be activated by voltage even in the absence of Ca^{2+}. BK channel β-subunits, several chemicals, and mutations can shift voltage activation toward more hyperpolarized potentials but in a rather complex manner. In contrast the accessory protein leucine-rich repeat containing protein 26 (LRRC26) and mallotoxin each appears to produce a simple shift. We have investigated the functional basis for the gating shift produced by mallotoxin in the absence and presence of the LRRC26 protein in the context of the Horrigan-Aldrich (HA) model. In the absence of intracellular Ca^{2+}, we found that, in addition to a hyperpolarizing shift of BK activation, mallotoxin produced a large, hyperpolarizing shift of channel activation kinetics. This result suggests that a major action of mallotoxin is to sensitize the BK channel voltage sensors. We also found that the degree of gating shift of mallotoxin was significantly decreased when co-expressed with the LRRC26 protein or in native parotid acinar cells that endogenously express this accessory protein. These results suggest that there is limit to the degree of sensitization of the BK channel voltage sensors.

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Cholesterol Regulates the Basal Functions and Ethanol Sensitivity of Large Conductance, Ca^{2+}-Sensitive K+ channel through Specific Cholesterol-Protein Interaction

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Membrane cholesterol plays an important role in regulation of a variety of ion channels and in maintaining the normal functions of cell membranes. The mechanism underlying this influence on ion channels, however, remains poorly understood. Cholesterol can act on ion channel proteins through either: 1) direct interaction with the protein, or 2) indirect effects on the biophysical properties of lipid bilayer. To differentiate between these alternatives, we used a synthesized enantiomer of cholesterol (ent-CHS). Enantiomeric forms of cholesterol have been used in the study of cholesterol-protein interaction, based upon the assumption that the effect on the lipid bilayer will be identical to that of natural cholesterol (nat-CHS), steric interaction directly with the protein will be abolished. We employed planar bilayer recording techniques to study the interaction of nat-CHS and ent-CHS with the large conductance, calcium-sensitive potassium channel (BK) in lipid bilayers of POPE/POPS (3/1, weight) and DOPE/SPM (3/2, weight), and examined how the presence of nat-CHS and ent-CHS affected the basal function and ethanol sensitivity of the BK channel. We found that ent-CHS increased BK channel conductance in both lipid bilayers similarly to that of nat-CHS. However, they are strikingly different in their effects on BK channel gating and ethanol sensitivity. In the POPE/POPS bilayer, nat-CHS dramatically reduced the open probability (Po) while ent-CHS did not. Both ent-CHS and nat-CHS reduced the ethanol sensitivity of the BK channel, but ent-CHS did so to a lesser extent. In the DOPE/SPM bilayer, nat-CHS dramatically changed the ethanol response of BK, depending on cholesterol concentration in the membrane. However, ent-CHS had little effect on ethanol sensitivity. We conclude that membrane cholesterol has a specific interaction with the BK channel that can directly influence ethanol's actions.

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The Slo1 C-Tail Domain Confers Cholesterol-Sensitivity to Arterial Smooth Muscle BK Channels

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Large conductance, voltage- and calcium-gated potassium (BK) channels are known to cluster in cholesterol-rich cell membrane domains. Furthermore, cholesterol-BK channel interaction usually results in reduced channel activity (Po), as reported with native (Bolotina et al., 1989) and recombinant (Crowley et al., 2003) channels. We previously communicated that cholesterol-inhibition of BK channels cloned from rat cerebral artery myocytes (cbv1; AY330293) and reconstituted into POPE:POPS (3:1) bilayers displayed enantio-specificity and stereoselectivity, strongly suggesting that the decrease in Po involved cholesterol-recognition by a protein surface (Bukiya et al., Biophys. Soc. 2010). Using a similar system, we now demonstrate that cholesterol (16-33 mol%) inhibition of cbv1 channels is similar whether the channel is open by positive voltage or increased intracellular calcium, suggesting that the cholesterol-protein(s) interaction leads to altered channel function independently of the signal that gates the channel. Because the channel phenotype was characteristic of homomeric slo1, we hypothesize that regulation of BK gating by cholesterol involves an interaction between the steroid and the cbv1 subunit. One of the motifs that favors cholesterol-protein interactions is CRAC: -L/V-(X)(1-5)-Y-(X)(1-5)-R/K-, where X denotes any residue (Epand, 2008). We used CRAC sequence patterning and found ten CRAC motifs in cbv1, three in the core (S0-S6) and seven in the C-tail (S7-C end) domain. Thus, we next evaluated the cholesterol sensitivity of cbv1 channels truncated immediately after S6 (trS6cbv1). Remarkably, trS6cbv1 was consistently resistant to cholesterol-induced modulation (n=11) under conditions where *wt* cbv1 remained sensitive. Therefore, the C-tail domain confers cholesterol-sensitivity to cbv1 channels. We are currently using a combination of computational dynamics, sequential cbv1 truncation, and point mutagenesis in CRAC motifs to determine the relative contribution, if any, of these motifs to the cholesterol-sensitivity of BK channels.

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A Functional Analysis of NaK at the Single Channel Level

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NaK is a non-selective monovalent cation channel from *Bacillus cereus*. Despite being unable to discriminate between Na^+ and K^+, NaK shows high sequence similarity to other K^+ channels. Based on recently solved crystal structures in the closed and putatively open state, NaK exhibits an overall architecture similar to that found in the pore domain of tetrameric K^+ channels. Rb^+ influx studies suggest the channel conducts cations, however net flux is unusually low for a channel. The absence of electrophysiological data from NaK precludes significant understanding of it's functional behavior. Using a random mutagenesis approach together with a K^+ transport based screen, we have identified gain-of-function mutants in an attempt to develop a system for electrophysiological studies. One of these purified and reconstituted mutants was further studied by liposome patch-clamp. The channel displays non-selective conductances at 25 and 91 pS and is characterized by a low probability spiking